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Functional effects of the antigen glatiramer acetate are complex and tightly associated with its composition

Tal Hasson [b,1], Sarah Kolitz [a,*,1], Fadi Towfic [a], Daphna Laifenfeld [b], Shlomo Bakshi [b], Olga Beriozkin [b], Maya Shacham-Abramson [b], Bracha Timan [b], Kevin D. Fowler [a], Tal Birnberg [b], Attila Konya [c], Arthur Komlosh [b], David Ladkani [b], Michael R. Hayden [b], Benjamin Zeskind [a,1], Iris Grossman [b,**,1]

[a] Immuneering Corporation, Cambridge, MA, USA
[b] Teva Pharmaceutical Industries, Petach Tikva, Israel
[c] Teva Pharmaceutical Works Ltd., Hungary

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ABSTRACT

Glatiramer acetate (Copaxone®; GA) is a non-biological complex drug for multiple sclerosis. GA modulated thousands of genes in genome-wide expression studies conducted in THP-1 cells and mouse splenocytes. Comparing GA with differently-manufactured glatiramoid Polimunol (Synthon) in mice yielded hundreds of differentially expressed probesets, including biologically-relevant genes (e.g. *Il18*, adj $p < 9e-6$) and pathways. In human monocytes, 700+ probesets differed between Polimunol and GA, enriching for 130+ pathways including response to lipopolysaccharide (adj. $p < 0.006$). Key differences were confirmed by qRT-PCR (splenocytes) or proteomics (THP-1). These studies demonstrate the complexity of GA's mechanisms of action, and may help inform therapeutic equivalence assessment.

1. Introduction

Glatiramer acetate (GA; Copaxone®) has been safely used to treat multiple sclerosis (MS) patients for almost two decades. Compositionally, it is a heterogeneous colloidal mixture of polymeric molecules, each up to 300 amino-acids long. Up to 10^{29} variants are present in the mixture, subsets of which exist at subpicogram quantities and thus remain unidentifiable with current state-of-the-art methodologies. Upon subcutaneous injection, GA is immediately hydrolyzed at the site of the injection, resulting in an undetectable systemic pharmacokinetic profile. Furthermore, the therapeutic properties of GA have yet to be correlated with any type of validated pharmacodynamic biomarker. Indeed, GA's active moiety(ies), ie, the specific amino acid sequences (acting effectively as immunological "epitopes", or antigenic motifs that uniquely activate certain aspects of the immune system) responsible for its clinical efficacy, have yet to be identified. Harnessing high resolution, comprehensive, and unbiased methods is essential to furthering the scientific understanding of the complex mode of action of GA.

The clinical effects of GA are believed to result from its functioning as an antigen modulating the immune system. GA was designed to mimic the autoantigen myelin basic protein (MBP), which is attacked by the immune system in multiple sclerosis. After degradation at the injection site, GA is thought to bind MHC Class II molecules on antigen-presenting cells (APCs), which interact with naïve T-cells, generating GA-specific T-cells and shifting their phenotype from pro-inflammatory helper-T types 1 and 17 (Th1/Th17) to anti-inflammatory regulatory T cells (Tregs) and helper-T type 2 (Th2) cells (Duda et al., 2000; Neuhaus et al., 2000; Arnon and Aharoni, 2004). GA-specific T-cells are able to migrate through the blood–brain barrier (BBB) and some of them cross-react with the similarly-structured MBP or other myelin associated antigens inducing local secretion of anti-inflammatory cytokines at the site of the lesions, shifting the balance from a pro-inflammatory (Th1/Th17) to anti-inflammatory (Th2/Treg) phenotype (Neuhaus et al., 2000; Arnon and Aharoni, 2004; Aharoni et al., 2010). GA also induces type-II monocytes, which direct differentiation of Th2 and protective Tregs (Kim et al., 2004; Weber et al., 2007), an effect that is independent of antigen specificity; thus cross-reactivity of T cells with myelin antigen is not required for therapeutic benefit (Weber et al., 2007). In addition, GA promotes production of neurotrophic factors such as BDNF by T cells (Arnon and Aharoni, 2004), and induces B-cell activation, which appears necessary for GA response in animal models (Jackson et al., 2014). Data from GA-treated MS patients indicate that GA also modulates CD8+ T cell

* Correspondence to: S. Kolitz, Immuneering Corporation, One Broadway, 14th Floor, Cambridge, MA 02142, USA.
** Correspondence to: I. Grossman, Teva Pharmaceutical Industries Ltd., 5 Basel St, Petach Tikva 49131, Israel.
E-mail addresses: skolitz@immuneering.com (S. Kolitz), Iris.Grossman@teva.co.il (I. Grossman).
[1] These authors contributed equally.

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Table 1
Numbers of differentially expressed probesets in each model system.

a) Mouse splenocyte data:

Immunization	Treatment comparison	Total	Up-regulated	Up, FC ≥ 2	# pathways	Down-regulated	Down, FC ≤ −2	# pathways
Copaxone	Copaxone-mannitol	16,647	8342	411	76	8305	485	56

Immunization	Treatment comparison	Total	Up-regulated	Up, FC ≥ 1.2	# pathways	Down-regulated	Down, FC ≤ −1.2	# pathways
Copaxone	(Polimunol-mannitol)-(Copaxone-mannitol)	223	208	73	22	15	6	0
Polimunol	(Polimunol-mannitol)-(Copaxone-mannitol)	431	301	77	10	130	22	0
Corresponding to treatment	$(\text{Polimunol-mannitol})_{\text{Pol}}$-$(\text{Copaxone-mannitol})_{\text{Cop}}$	462	362	206	25	100	30	3

b) THP-1 data:

	Treatment comparison	Total	Up-regulated	Up, FC ≥ 1.3	# pathways	Down-regulated	Down, FC ≤ −1.3	# pathways
Pre-filtering	Copaxone-mannitol	12,115	5296	456	180	6819	183	6
Post-filtering	Copaxone-mannitol	12,001	5227	456	180	6774	183	6

	Treatment comparison	Total	Up-regulated	Up, FC ≥ 1.1	# pathways	Down-regulated	Down, FC ≤ −1.1	# pathways
Pre-filtering	(Polimunol-mannitol)-(Copaxone-mannitol)	807	518	241	177	289	80	0
Post-filtering	(Polimunol-mannitol)-(Copaxone-mannitol)	779	494	218	137	285	78	0

activity (Karandikar et al., 2002). Additional mechanisms may also be involved in GA action.

The complexity of GA's mechanism of action (MoA) cannot be fully captured by any one in vitro or in vivo system. Genome-wide expression profiling of splenocytes extracted from GA-treated mice serves to capture elements of the biological impact in T cells, given the observed physicochemical differences between GA and other glatiramoids (Weinstein et al., 2015). This model system has demonstrated utility as a means to model GA's interaction with lymphocytes (Bakshi et al., 2013; Towfic et al., 2014); and similar studies in a human monocyte cell line have been used to study GA's impact on APCs (Kolitz et al., 2015). Together, these studies represent all three key elements required for GA's activation of "the immunological triad" — including lymphocytes, immunogenic epitopes, and APCs (Jackson et al., 2014; Sellebjerg et al., 2013), while also accounting for some of the factors that further modulate therapeutic response in vivo. Utilizing learnings from previous studies of GA in these cell types, a reciprocal-control experimental design was applied in order to further elucidate GA's MoA and to assess the degree to which these mechanisms are sensitive to differences in composition and manufacturing of the therapeutic antigen. Namely, mouse splenocytes were utilized to model three likely clinical scenarios in a single experiment. The first scenario models a patient treated initially with GA and then switched to a follow-on glatiramoid (FOGA), which is modeled by immunizing mice with GA and then comparing the gene expression profiles when extracted splenocytes are activated ex vivo with either GA or FOGA. The second scenario is one in which a patient is treated initially with a FOGA and then switched to GA, which is modeled by immunizing mice with FOGA and then comparing the gene expression profiles when extracted splenocytes are activated ex vivo with either GA or FOGA. The third scenario models a setting in which a patient is treated purely with either GA or FOGA, such that comparison of the gene expression profiles derived from the activated splenocytes in each experiment may reflect long-term treatment-induced functional differences. This experimental design was combined with mRNA and proteomic studies conducted with the same glatiramoids in a human monocyte cell line, previously published as consistent with human primary monocyte studies in glatiramoids (Kolitz et al., 2015). Taken together, these orthogonal experimental models, biomarker technologies, and functional analyses provide important insights into GA's mode of action and differences with FOGAs.

The analysis of multiple lots of GA was conducted in parallel to Synthon's FOGA (Polimunol®), marketed in Argentina as a purported clinical equivalent to GA (as of May 2014). Polimunol is believed to be the same Synthon FOGA (entitled "GTR") used in the only bridging clinical trial conducted to date with the objective of demonstrating surrogate equivalence to GA in terms of MRI measurements (GATE study, NCT Number NCT01489254). The authors report that for T1-GdE lesions during months 7–9 the "GTR/GA ratio was within the pre-defined margins". However, according to results reported from the placebo-controlled phase of the study (Cohen et al., 2014), clinical equivalence in terms of annualized relapse rate (ARR), as well as correlation between MRI-lesion predictors and ARR reductions, have not been met, indicating lack of sameness in therapeutic effect. It is for these reasons that studies comparing Synthon's FOGA to GA using high-resolution physicochemical tests, as well as genome-wide expression studies in immunologically relevant model systems, are of keen interest to the scientific and medical community worldwide, considering that no traditional therapeutic equivalence methods have been shown to be clinically validated for this class of compounds. Thus, careful investigation and establishment of novel preclinical and clinical standards are needed in evaluating therapeutic equivalence between FOGAs and GA.

2. Materials and methods

2.1. Reciprocal mouse splenocyte study: experimental design and methods

2.1.1. Mice

All experimental procedures conformed to accepted ethical standards for use of animals in research and were in accordance with Committee for the Care and Use of Experimental Animals guidelines and approved by the Teva Institutional Animal Care and Use Committee. 8- to 12-week-old female (Balb/c X SJL) F1 mice (Janvier, France) were purchased, and kept at 21 ± 3 °C with relative humidity 30%–70%, and light/dark cycle 12:12 h. Animals were maintained on a standard rodent pellet diet and sterile filtered tap water available ad libitum.

2.1.2. Immunization of mice and preparation of ex vivo spleen cell cultures

To stimulate induction of GA and FOGA-reactive T cells, twelve mice in each treatment group were injected subcutaneously with 100 μL of a 2.5 mg/mL solution of either Copaxone (GA drug product, Teva Pharmaceutical Industries, Petach Tikva, Israel) or Polimunol (FOGA drug product, Synthon, Nijmegen, Netherlands) in phosphate-buffered saline (250 μg GA per mouse). Mice were housed for 3 days after immunization, then sacrificed. Spleens were aseptically removed and placed on ice in RPMI 1640. A single cell suspension was prepared. After red blood cell lysis, splenocytes from the same immunization group were pulled and resuspended to a final concentration of 10×10^6 cells/mL in defined cell culture medium (DCCM1) (Biological Industries, Beit

Haemek, Israel) (96.7% *v*/*v*) enriched with L-glutamine 2 mM (1% *v*/*v*), MEM Non-Essential Amino Acids 2 mM (1% *v*/*v*), sodium pyruvate 1 mM (1% *v*/*v*), antibiotic/antimycotic solution (0.2% *v*/*v*) and 2-mercaptoethanol 50 mM (0.1% *v*/*v*).

2.1.3. In vitro cell activation

Splenocytes were treated with activator samples diluted in medium (125 μL per well of 80 μg/mL solutions, final concentration in the well after addition of cells: 40 μg/mL) of: (i) 3 different lots of GA drug product manufactured by Teva, or (ii) one lot of FOGA (Polimunol). The activator samples or mannitol (the nonactive excipient in GA/FOGA drug products, used as control in the experiment) in medium were added to 96-well tissue-culture plates (three wells per sample). Splenocytes (125 μL 10×10^6 cell/mL suspension) were added to the activator solutions. Each activator sample was loaded in 2 different plates, 1 for cells from mice immunized with GA and 1 for cells from mice immunized with purported generic. Plates were incubated for 24 h at 37 °C in 5% CO_2 atmosphere. Cells were collected and centrifuged at 300 *g* for 5 min. Supernatants were aspirated and cell pellets resuspended in RLT buffer (from RNeasy mini kit of Qiagen, Cat # 74106) for cell lysis. Cell lysates were centrifuged and supernatants collected and frozen at −70 °C. Samples were sent for further processing. RNA was extracted and expression-profiled across the entire genome using the Affymetrix Mouse Genome 430_2 chip (Expression Analysis|Quintiles, Durham NC). Three lots of GA and 1 lot FOGA were comparatively tested in 6 biological replicates each, as dictated by power-analysis calculations performed using the R statistical package ssize.fdr to determine the number of samples needed to detect differentially-expressed genes with a fold-change between treatments of as low as 1.3 with 80% power. Two technical replicates were used, resulting in 12 replicates per condition.

2.1.4. qRT-PCR confirmation

Ten genes (*Il10*, *Il4*, *Foxp3*, *Il18*, *Mmp8*, *Rsad2*, *Ddx58*, *Irf7*, *Mx1*, *Ifi202b*) were chosen for testing based on showing significant modulation in the microarray observations as well as potential biological interest. Experimental assistance was provided by EA/Quintiles. Expression levels of the ten genes were measured using qRT-PCR with 18S rRNA as reference transcript, and mannitol samples as calibrator. Relative quantification (RQ) values were computed as $2^{-\Delta\Delta Ct}$. Differences in expression levels were evaluated for significance using 2-sided *t* tests with equal variance.

2.2. THP-1 microarray study experimental design and methods

Cells from a human monocyte cell line (THP-1) were stimulated with either branded GA drug product, FOGA (Polimunol), or vehicle control (mannitol) for 6 h based on prior observations of GA effects in this model system (Kolitz et al., 2015). RNA was extracted and expression-profiled in a blinded fashion across the entire genome, using the Affymetrix Human Genome U133 Plus 2.0 chip, interrogating a total of over 47,000 transcripts (Expression Analysis|Quintiles, Durham NC). Three lots of GA and 1 lot of FOGA were comparatively tested in 6 biological replicates each. This entire experiment was performed independently twice, using an identical study design, reagents and compounds by the same technicians on 2 separate days; resulting in 12 biological replicates total per condition.

2.3. THP-1 proteomic study experimental design and methods

Cells from the same human monocyte cell line (THP-1) were incubated with either branded GA, FOGA Polimunol, or vehicle control (mannitol) for 24 h, expected to reflect translation of mRNA expression patterns observed in the same study design at 6 h post stimulation. Supernatants (1.0 mL of cell culture media) were collected and assayed for the concentration of selected proteins using a custom Luminex assay to measure the concentrations of a panel of 42 chemokines and cytokines (in pg/ml) using Bio-Plex Human Chemokine (Bio Rad kit) and Luminex Performance Assay (R&D kit). Five biological replicates per condition were used.

2.3.1. Data analysis for proteomic data

Data (concentrations in pg/mL) were compared using a 2-sided *t* test with equal variance and corrected for multiple hypothesis testing using Benjamini–Hochberg correction. To calculate the fold change between the protein expression levels with FOGA and with GA, GA values were averaged together and compared with the average value for Polimunol (average Polimunol expression level/average GA expression level).

2.4. A priori power analysis

Using the R statistical package ssize.fdr, power calculations were performed to determine the number of samples needed to detect differentially-expressed genes with a fold-change between treatments of as low as 1.3 with 80% power. Based on the results of these power calculations, the experiment was designed to include 6 biological replicates of each condition. The order in which samples were processed was randomized with respect to treatment and stimulation time to avoid creating confounding batch effects.

2.5. Analysis methods for microarray studies

2.5.1. Outlier identification

Outlier samples were identified using the R package ArrayQCMetrics and excluded from further data processing steps. A sample was considered an outlier if it failed more than half of the included tests either before or after RMA normalization. For mouse splenocyte data, 6 samples (3.8%) were determined to be outliers, and for THP-1 data, 2 samples (2.4%) were determined to be outliers by these criteria. Data were RMA normalized using the Affy R package.

2.5.2. Batch correction

Correction for batch variation was performed using ComBat (Johnson et al., 2007), as implemented in the SVA R package (Leek et al., 2013). Briefly, ComBat is an empirical Bayesian approach using location and scale metrics across several genes to adjust for batch effects in datasets, even datasets containing small sample sizes. For mouse splenocyte data, date of microarray experiment was used as batch, and the combination of treatment and immunization was used as covariate. For THP-1 data, date of experiment was utilized as batch and treatment labels were added as covariates to the batch correction to preserve relevant treatment effects. Principal Component Analysis (a multivariate approach) showed that the main effect in the first principal component remained due to treatment effects after batch correction.

2.5.3. Differential expression analysis

Differentially-expressed probesets were identified across conditions using linear models for microarray data (LIMMA) (Smyth, 2004). Comparisons of GA and purported generic were corrected to compare each treatment relative to mannitol control (e.g., [GA vs mannitol] was compared via LIMMA with [Polimunol vs mannitol]). Probesets were filtered by MAS5 calls of presence on the chip (to be considered present, a probeset was required to have on average a call of present or marginal across samples).

For mouse splenocyte studies, probesets were mapped to genes using the annotation available for the Mouse_430_2 chip from Affymetrix. For the comparison of immunization and activation with GA versus immunization and activation with FOGA, an additional correction for run order, performed using LIMMA (Smyth, 2004), was evaluated (Supplementary Table 2).

For THP-1 studies, an additional QC step was performed to remove probesets highly variable between THP-1 datasets, as follows: a probeset was deemed highly variable if across three THP-1 studies to date, that probeset was observed to be upregulated, downregulated, and not modulated by GA across the three studies. This criterion resulted in filtering out 216 probesets. Probesets were mapped to genes using the annotation available for the U133 Plus 2.0 chip from Affymetrix.

Unless otherwise noted, FDR-adjusted p values reported for genes represent the lowest FDR-adjusted p value for present probesets for that gene.

2.5.4. Pathway enrichment analysis

Upregulated and downregulated probesets were analyzed separately for pathway enrichment, using DAVID (Huang et al., 2009). The background used was probesets with present calls on the chip. Results were visualized using volcano plots, plotting −log adjusted p values versus enrichment scores for the pathways. Fold-change (FC) cutoffs were utilized to obtain lists of probesets of a size appropriate for the step of pathway enrichment using DAVID. For comparisons between GA and Polimunol, upregulated or downregulated probesets with FDR-adjusted p values < 0.05 and fold changes with absolute value ≥ 1.2 (for mouse splenocyte study) or 1.1 (for THP-1 study) were used. For comparisons between GA and mannitol, upregulated or downregulated probesets with FDR-adjusted p values < 0.05 and FC with absolute value greater than 2 (mouse splenocyte study) or 1.3 (THP-1 study) were used. DAVID runs were conducted in December 2014 and January 2015.

3. Results

3.1. Reciprocal mouse splenocyte studies

3.1.1. GA mechanism of action

Analyzing splenocytes from mice immunized as well as activated with GA, 16,647 probesets were significantly modulated relative to mannitol: 8342 (representing 5101 genes) increased in expression level (throughout, termed upregulated) and 8305 (5208 genes) decreased in expression level (throughout, termed downregulated). Filtering by |FC| ≥ 2, 411 probesets (271 genes) were upregulated and 485 (355 genes) downregulated (Table 1).

As expected, GA treatment upregulated key anti-inflammatory cytokine genes *Il10* and *Il4* (adj p < 2.3e−24 and 5.1e−35, respectively; Fig. 1a), as well as markers of regulatory T cells, *Foxp3* and *Gpr83* (adj p < 3.4e−23 for *Foxp3*; Fig. 1a; adj p < 4.2e−33 and 9.0e−31 for the 2 *Gpr83* probesets). Complementarily, GA downregulated pro-inflammatory cytokine genes such as *Il12a* (adj p < 8.3e−31; Fig. 1b).

After imposing a conservative fold change filter of |FC| ≥ 2, 411 probesets were upregulated by GA relative to mannitol (in GA-immunized mice). These probesets enriched for 76 pathways, many of

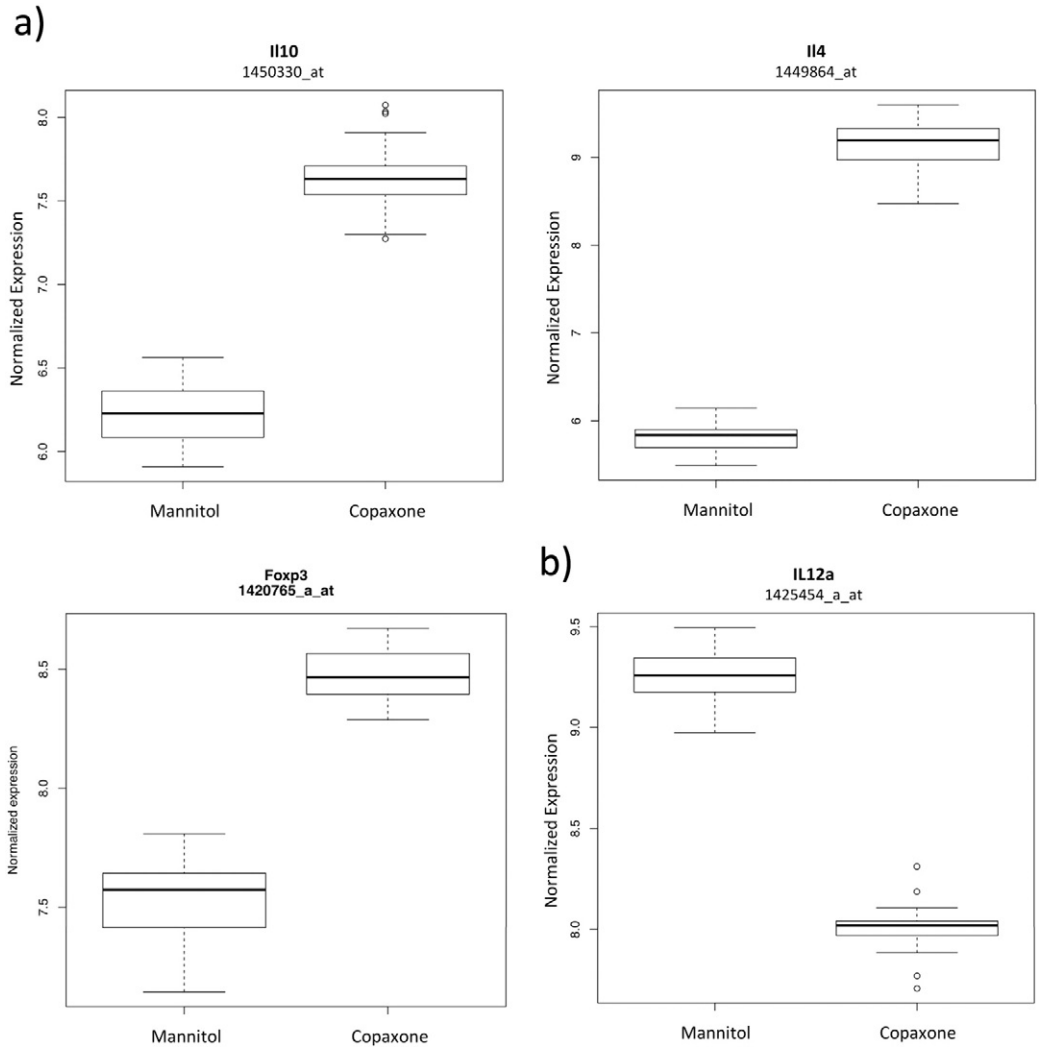


Fig. 1. In splenocytes from mice immunized with GA, GA treatment increased expression of the genes for anti-inflammatory cytokines IL-10 and IL-4 and regulatory T cell marker FOXP3 (a), and decreased expression of the gene for the pro-inflammatory cytokine IL-12a (b).

which are immunologically relevant, and include relevant aspects of GA's MoA such as the cytokine–cytokine receptor interaction pathway identified also in the THP-1 data described below. Similarly, after filtering by |FC| ≥ 2, 485 downregulated probesets were detected, enriching for 56 pathways, a number of which are associated with modulation of the immune system. Both the upregulated and downregulated pathways are depicted in Fig. 2, and the full list is provided in Supplementary Table 1.

3.1.2. GA versus differently-manufactured FOGA Polimunol

First, the "switching" model was utilized to detect genomic modulation associated with activation by a differently manufactured FOGA than the immunization agent, GA. This model is expected to emulate the clinical situation of FOGA introduction in the market and forced-switching of GA-treated patients to FOGA mid-treatment.

When splenocytes from GA-immunized mice were activated ex vivo with either FOGA or GA and compared to each other while controlling for mannitol, 223 probesets were found to be differentially expressed at FDR $p < 0.05$ (208 were upregulated, 15 were downregulated). The top upregulated probesets by FOGA versus GA are reported in Supplementary Table 3 and include many interferon-induced genes, as detailed in the pathway enrichment section below. The 15 downregulated probesets by FOGA versus GA are reported in Supplementary Table 4.

Next, the reciprocal experiment was carried out, conducting the immunization with FOGA rather than GA. When splenocytes from FOGA-immunized mice were activated with GA versus activation by the same FOGA and controlling for mannitol, 431 probesets were differentially expressed (adj $p < 0.05$; 301 upregulated, 130 downregulated). The top upregulated probesets by FOGA versus GA are reported in Supplementary Table 5. This list overlaps greatly (149 of 208 = 72%) with probesets upregulated in the reciprocal comparison described above (ie, same study design except immunization agent being GA rather than FOGA). This consistency is highly statistically significant ($p < 1e-10$ by hypergeometric test) and includes many interferon-induced genes. Similar observations for downregulated probesets by FOGA versus GA were obtained, and the top results are reported in Supplementary Table 6.

The genes depicted as differentially modulated by FOGA compared with GA are linked to clinically relevant functionality, specifically as relates to treatment of multiple sclerosis. For instance, while *Il18* and its receptor *Il18r1* were both significantly downregulated by the 2 activation treatments, regardless of immunization agent (GA or FOGA), *Il18* was downregulated significantly less by FOGA than by GA (differential expression adj $p < 9e-6$, FC = 1.20 under GA immunization, and adj $p < 2e-9$, FC = 1.26 under FOGA immunization). Boxplots of *Il18* expression appear in Fig. 3a.

After imposing a fold change filter of |FC| ≥ 1.2 and adjusting p value for multiple testing, 73 probesets were found to be upregulated by Polimunol relative to GA (mannitol-corrected, in GA-immunized mice), enriching for 22 pathways, including immune response and defense response. Several of these pathways may be relevant to GA's MoA, since they were also enriched among probesets modulated by GA relative to control (e.g., immune response, immune system process, and defense response). Additional pathways, including response to virus, RIG-I-like receptor signaling, and cytosolic DNA-sensing pathway, are consistent with increased type I interferon signaling. A total of 6 downregulated probesets were detected, which do not enrich for any known pathway. The upregulated pathways are depicted in Fig. 4a and the full list of pathways in Supplementary Table 7.

The same analysis conducted in the reciprocal study design with Polimunol-immunization (imposing filters of |FC| ≥ 1.2 and adjusted $p < 0.05$), 77 probesets were upregulated significantly by Polimunol relative to GA (mannitol-corrected), and these probesets enriched for 10 pathways. The majority of the pathways overlapped with those seen in the comparison for GA-immunized mice, and include pathways relevant to GA's MoA such as immune response and immune system process pathways. As with GA immunization, the response to virus pathway was also enriched. Twenty-two downregulated probesets were detected, which did not enrich for known pathways. The upregulated pathways are depicted in Fig. 4b and provided in Supplementary Table 8.

3.1.3. Comparison of single-agent immunization and activation sequence with GA versus Polimunol

Gene expression profiles from splenocytes of GA-immunized mice treated ex vivo with GA were compared with gene expression profiles from splenocytes of Polimunol-immunized mice treated ex vivo with Polimunol. 235 probesets were differentially expressed after imposing a fold change filter of |FC| ≥ 1.2 and correction for multiple hypothesis testing (FDR p value < 0.05); 206 of those were upregulated and 30

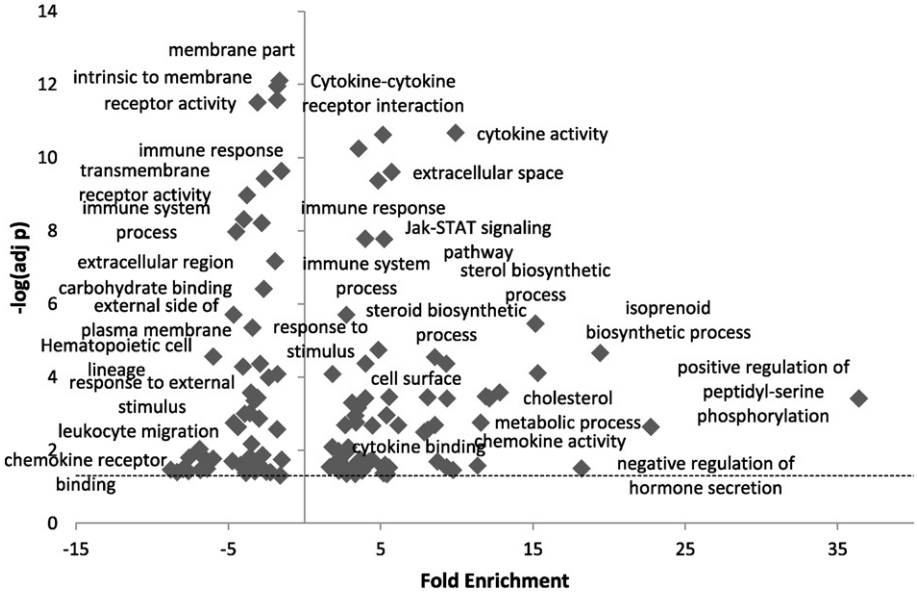


Fig. 2. Pathways enriched among top probesets modulated by GA relative to mannitol in splenocytes from mice immunized with GA. The dashed line indicates significance level of adjusted p value < 0.05.

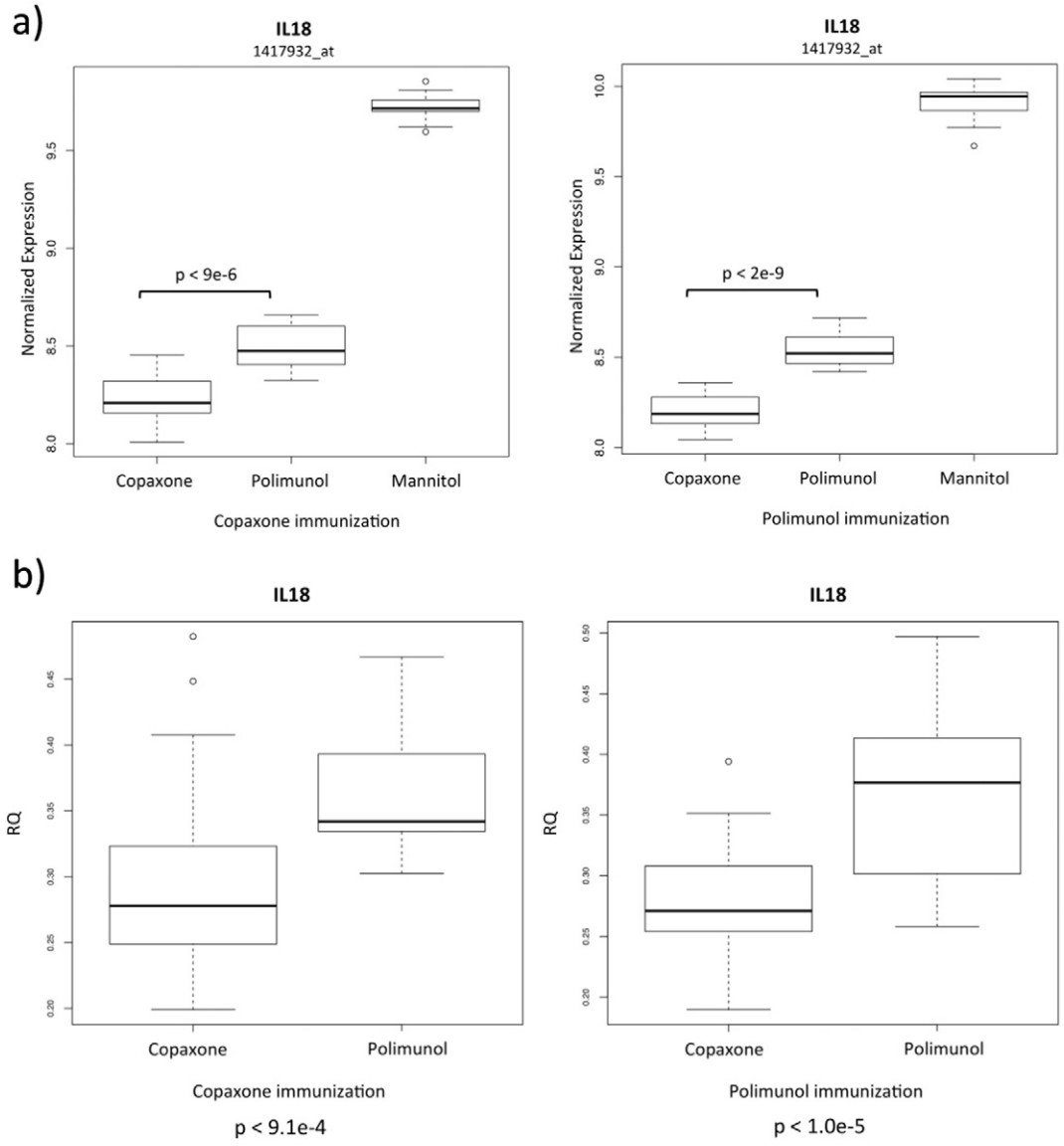


Fig. 3. Il18 Expression is reduced to a greater extent by GA than Polimunol, regardless of which substance is utilized for the immunization, both in microarray data (a) and qRT-PCR confirmation (b).

were downregulated in the Polimunol case versus the GA case. The upregulated probesets enriched significantly for 25 pathways, including response to virus (adj $p < 3.6e-9$) and RIG-I-like receptor signaling (adj $p < 0.004$). These observations were consistent at the gene and pathway level with the results of comparing Polimunol versus GA treatment within GA-immunized mice (Supplementary Table 9, Supplementary Fig. 1). In addition, the downregulated probesets enriched significantly for 3 pathways, including transmission of nerve impulse (adj $p < 0.04$) (Supplementary Fig. 1, Supplementary Table 9).

3.1.4. qRT-PCR confirmation of key results

Ten genes (*Il10*, *Il4*, *Foxp3*, *Il18*, *Mmp8*, *Rsad2*, *Ddx58*, *Irf7*, *Mx1*, *and Ifi202b*) were tested by qRT-PCR, to confirm the microarray findings for the above analyses. As expected (Supplementary Fig. 2a, Supplementary Table 10) in follow-up of GA mode-of-action genes, *Il10*, *Il4*, and *Foxp3* were confirmed as upregulated significantly by GA ($p < 1.3e-13$, $p < 7.4e-18$, and $p < 4.8e-13$, respectively).

As shown in Supplementary Table 10, the qRT-PCR results also confirmed microarray observations for differences between GA and Polimunol. Each of the 7 genes tested due to differing expression level by Polimunol versus GA treatment on the microarray also differed significantly in qRT-PCR, for each immunization condition.

Confirming the microarray results, *Il18* was significantly upregulated with Polimunol treatment relative to GA treatment, irrespective of immunization condition (either Polimunol or GA; $p < 9.2e-4$ and $p < 1.0e-5$, respectively) (Fig. 3b), reflecting the observation that Polimunol downregulated *Il18* to a significantly lesser extent than GA. For each of the other 6 genes, GA treatment increased the expression level relative to mannitol control, and Polimunol increased this expression level significantly further.

Since several genes from the RIG-I pathway were significantly upregulated by Polimunol compared with GA on the microarray, a few were chosen for qRT-PCR testing, including *Ddx58* (RIG-I) and *Irf7*. Confirmation was obtained for both immunization conditions (Supplementary Fig. 2b). A number of other interferon-related genes were also significantly modulated by Polimunol relative to GA (Supplementary Table 8), including *Mx1* and *Rsad2*, which have been used or proposed as markers of IFNβ response in multiple sclerosis (Hundeshagen et al., 2012; Malhotra et al., 2011). *Mmp8*, a gene that has been implicated in EAE (Folgueras et al., 2008), was also expressed more highly with Polimunol versus GA treatment (Supplementary Fig. 2b).

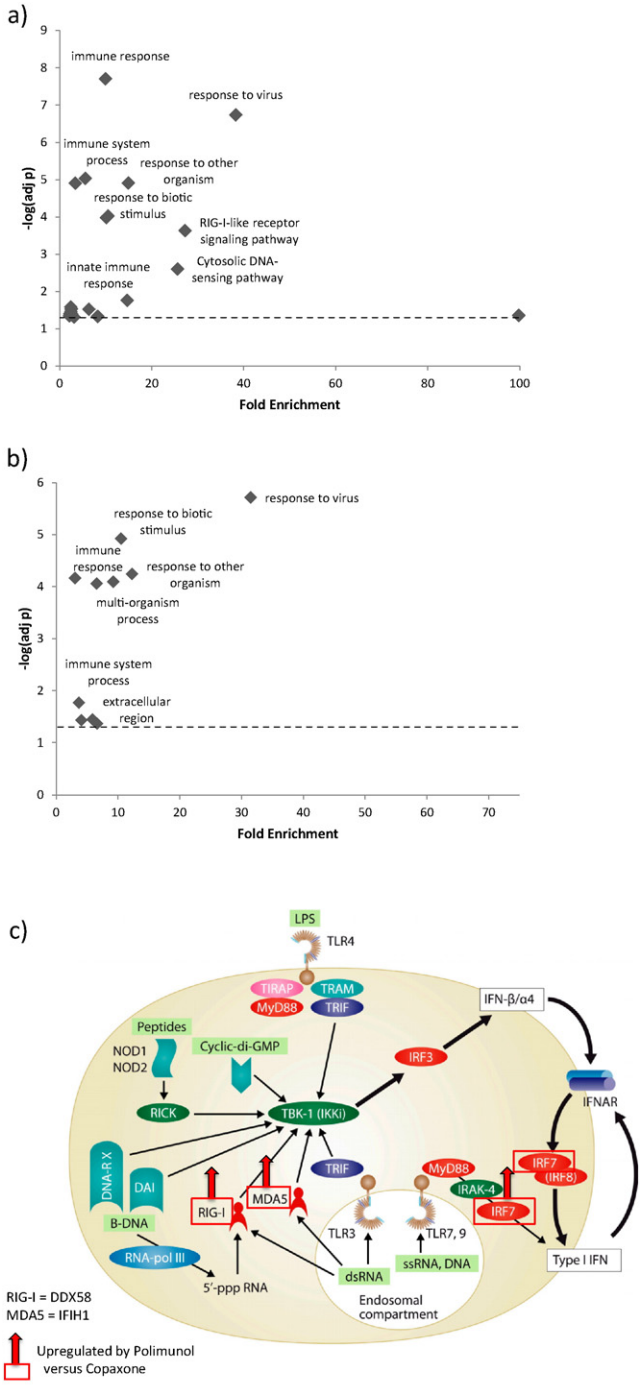


Fig. 4. Pathways enriched among top probesets upregulated by Polimunol relative to GA, in GA-immunized mice (a) and Polimunol-immunized mice (b). The dashed line indicates significance level of adjusted p value < 0.05. (c) Pathways of Type I interferon production (adapted from (Trinchieri, 2010): ©2010 Trinchieri. Journal of Experimental Medicine. 207:2053-2063. http://dx.doi.org/10.1084/jem.20101664).

3.2. THP-1 studies

3.2.1. GA mechanism of action

GA significantly upregulated 5296 probesets (3164 genes), and significantly downregulated 6819 probesets (4586 genes), out of >25,000 probesets called present on the chip. Consistent with previous studies (e.g., Kolitz et al., 2015), the anti-inflammatory gene *IL1RN* was strongly upregulated by GA (all 3 *IL1RN* probesets that were called present in the study: adj p values < 6.2e−21, 2.6e−17, 8.0e−14); see Fig. 5a.

IL10 was represented by a single probeset on the chip, which was not called "present", thus its modulation could not be determined. *IL10RA*, representing the receptor necessary for *IL10* signaling, was significantly upregulated (adj p < 1.7e−21), indicating this pathway upregulated by GA, as expected.

Among the top probesets upregulated by GA, 180 pathways were enriched (Fig. 5b, Supplementary Table 11). This includes immune response, immune system process, cytokine–cytokine receptor interaction, regulation of B cell activation, coagulation, positive regulation of lymphocyte activation and proliferation, and positive regulation of B-cell activation and proliferation. As an example of an enriched pathway anticipated as relevant to GA MoA, cytokine–cytokine receptor interaction, the individual genes modulated by GA involved in this pathway are depicted in Supplementary Fig. 3. Six pathways were enriched among top probesets downregulated by GA, including developmental processes, and cell–cell adhesion-related pathways. Many of the above pathways were observed in a prior study (Kolitz et al., 2015).

3.2.1.1. Secreted proteins modulated by GA. To evaluate effects of GA at the protein level, 42 secreted cytokines and chemokines were measured from the supernatant of THP-1 cells stimulated for 24 h with GA or mannitol control. Compared with mannitol control, GA significantly increased the levels of most of these proteins, including 34 out of 36 total proteins having sufficient measurements for any testing. None of the measured protein levels were decreased by GA.

3.2.2. Comparison of GA with Polimunol

Comparing Polimunol with GA, corrected for mannitol control expression [ie, (Polimunol-mannitol)-(GA-mannitol)], resulted in 807 differentially-expressed probesets with FDR-adjusted p < 0.05: 518 upregulated and 289 downregulated.

After conservative filtering of highly variable probesets (ie, the few previously identified to have differing behavior between THP-1 studies, which removed 3.5% of the 807 probesets), 779 modulated probesets persist: 494 upregulated probesets and 285 downregulated probesets (Supplementary Tables 12–13 list the top 25 probesets in each direction).

Polimunol consistently upregulated *CYP1B1* relative to GA (all 4 probesets on chip: adj p values 4.5e−11, 3.6e−9, 1.6e−8, 1.1e−7; see Fig. 6a). Also, relative to GA, Polimunol upregulated genes involved in inflammatory pathways, including *IL1B*, *MMP9*, *CCL2*, *CCL5*, and *CXCL1* (adj p values 1.2e−7, 2.0e−4, 2.7e−7, 0.01, and 4.3e−5, respectively).

Using the DAVID platform, 137 pathways were enriched among top probesets (obtained via filtering both as described above and by FC as described in Section 2) upregulated by Polimunol relative to GA. These pathways include a variety of immune-related pathways expected to be relevant to GA's MoA, including the cytokine–cytokine receptor interaction pathway (adj p < 1.9e−5) that was also enriched among probesets modulated by GA (see previous section), and positive regulation of cytokine production (adjusted p < 0.004). These pathways also include inflammation related pathways, such as inflammatory response (adjusted p < 0.001), NOD-like receptor signaling (adjusted p < 0.02), and response to lipopolysaccharide (adjusted p < 0.006). The top 25 pathways are listed in Supplementary Table 14, and the key pathways illustrated in Fig. 6b. The specific genes in the response to lipopolysaccharide pathway are depicted in Fig. 6c, and the genes in the cytokine–cytokine receptor interaction pathway are depicted in Supplementary Fig. 4.

3.2.2.1. Secreted proteins modulated by FOGA relative to GA. To evaluate differences between GA and FOGA at the protein level, a custom panel of 42 secreted cytokines and chemokines were measured from the supernatant of THP-1 cells stimulated for 24 h with GA, FOGA, or mannitol

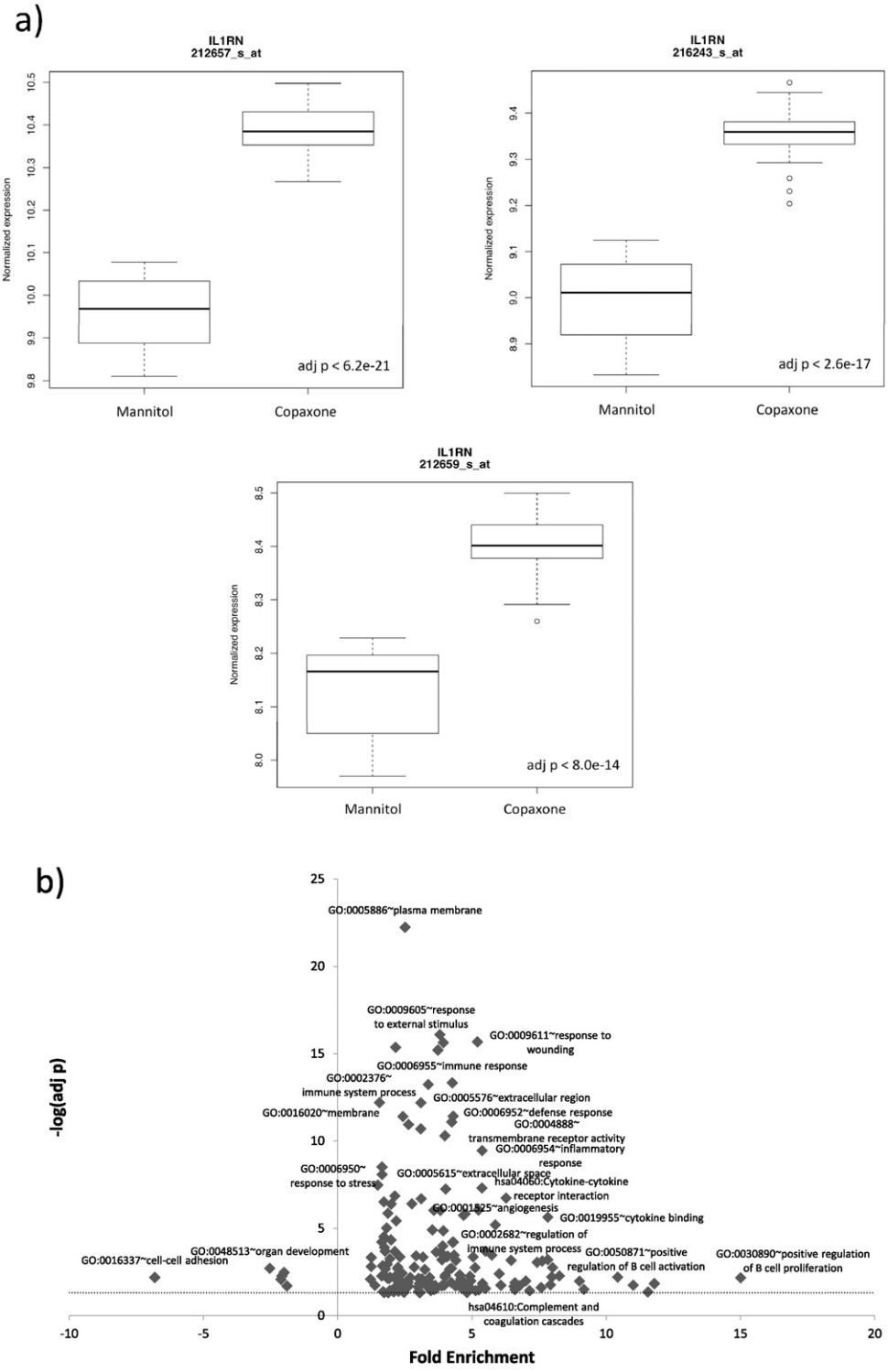
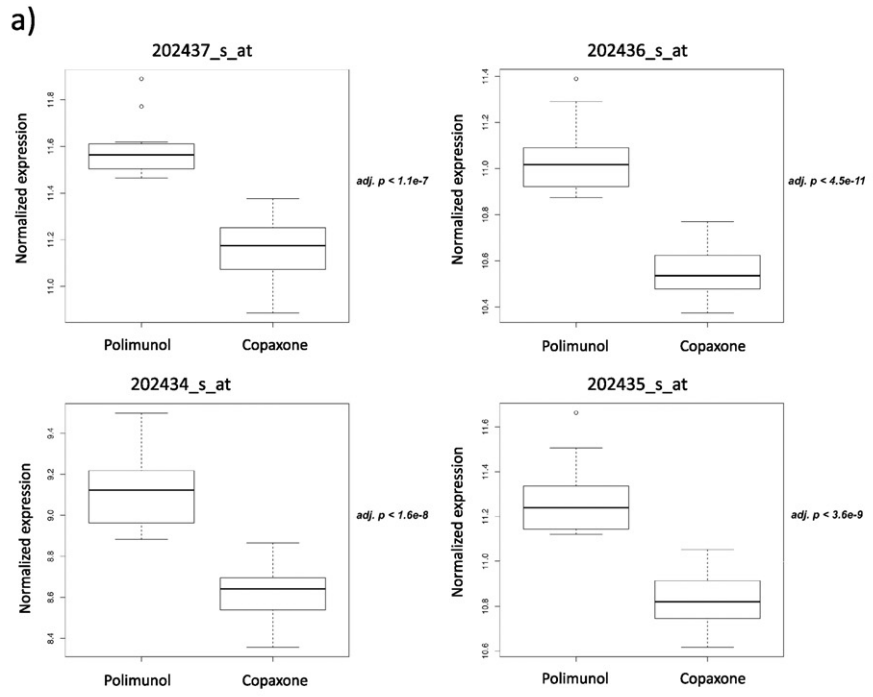


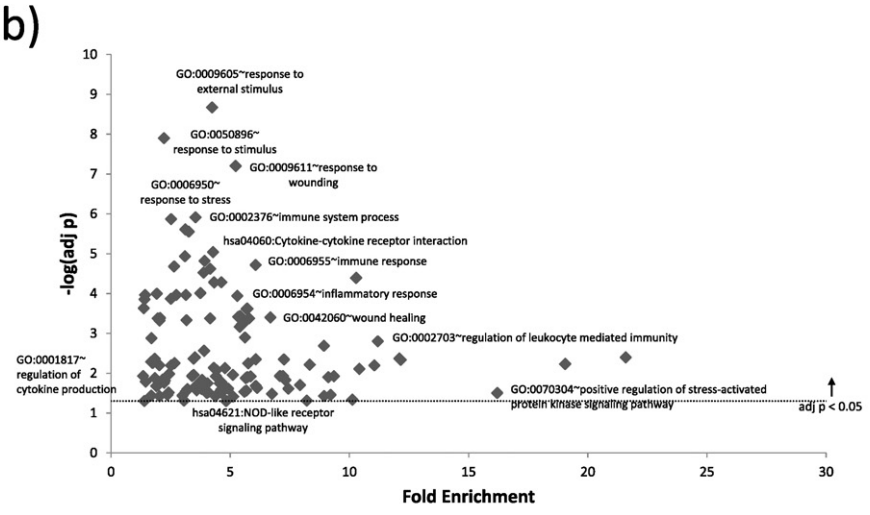
Fig. 5. Anti-inflammatory gene IL1RN is significantly upregulated by Copaxone (a); pathways enriched among top probesets modulated by Copaxone relative to mannitol control (b).

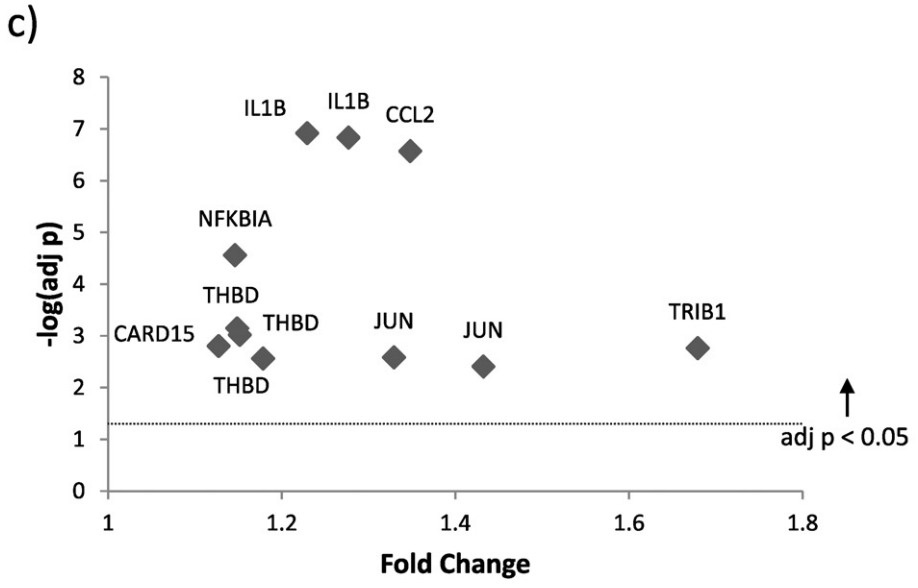
control. Comparing FOGA versus GA, none of the measured protein levels were lowered by FOGA treatment relative to GA. FOGA treatment significantly increased the levels of 23 proteins (out of 39 having sufficient measurements for testing; Supplementary Table 15). These include IFNg, TNFa, MIP-1a (CCL3), IL-8 (CXCL8), and IL-10. Consistent with the result observed at the mRNA expression level discussed above, secreted levels of MMP-9, MCP-1 (CCL2), RANTES (CCL5), Gro-a (CXCL1), and IL-1b were increased with FOGA treatment relative to GA (Fig. 7; adjusted p values as shown).

4. Discussion

T cells and antigen-presenting cells (APCs) are the two critical components that together with GA (or the autoantigen) comprise the immunogenic triad responsible for aspects of the antigenic MoA of GA. A comprehensive analysis of both cell types has been pursued, and follow-up experiments further confirmed the validity of findings, both in terms of technological platform (genome-wide expression profiles confirmed by qRT-PCR), and biologically (protein secretion levels


a)
202437_s_at
Normalized expression
adj. p < 1.1e-7
Polimunol
Copaxone
202436_s_at
Normalized expression
adj. p < 4.5e-11
Polimunol
Copaxone
202434_s_at
Normalized expression
adj. p < 1.6e-8
Polimunol
Copaxone
202435_s_at
Normalized expression
adj. p < 3.6e-9
Polimunol
Copaxone


b)
-log(adj p)
Fold Enrichment
GO:0009605~response to external stimulus
GO:0050896~ response to stimulus
GO:0009611~response to wounding
GO:0006950~ response to stress
GO:0002376~immune system process
hsa04060:Cytokine-cytokine receptor interaction
GO:0006955~immune response
GO:0006954~inflammatory response
GO:0042060~wound healing
GO:0002703~regulation of leukocyte mediated immunity
GO:0001817~ regulation of cytokine production
GO:0070304~positive regulation of stress-activated protein kinase signaling pathway
hsa04621:NOD-like receptor signaling pathway
adj p < 0.05


c)
-log(adj p)
Fold Change
IL1B
IL1B
CCL2
NFKBIA
THBD
CARD15
THBD
THBD
JUN
JUN
TRIB1
adj p < 0.05

confirmed relevant mRNA findings). An animal model was used in a parallel, reciprocal-control experimental design. Gene-level and pathway-level analyses identify known and novel modulatory effects of GA associated with its therapeutic effect, and reveal functionally relevant differences between GA and FOGA Polimunol that may shed light on clinical effects in multiple sclerosis patients.

As one approach to modeling T-cell relevant effects, a mouse splenocyte system was used, combined with a reciprocal experimental design which enabled the study of 3 scenarios: switching from GA to Polimunol, switching from Polimunol to GA, and comparing the impact of consistent GA use with the impact of consistent Polimunol use. The immunized mouse splenocyte system has been previously proven to be informative in examining GA's mode of action, as well as in differentiating between seemingly similar differently-manufactured GAs and GA (Bakshi et al., 2013; Towfic et al., 2014) in GA immunization scenarios, and the reciprocal experimental design used herein greatly expands the sensitivity and specificity of the overall functional insights associated with unique attributes of GA and as compared with differentially manufactured FOGAs.

More than two thirds of the probesets expressed in the spleen were significantly modulated by GA treatment, and the top modulated probesets in either direction were enriched for many relevant pathways, including immune response and cytokine–cytokine receptor interaction pathways. Key anti-inflammatory cytokine genes, such as *Il10* and *Il4*, and regulatory T cell markers, such as *Foxp3* and *Gpr83*, are upregulated by GA, while pro-inflammatory cytokine gene *Il12a* is downregulated by GA treatment in this model system, consistent with induction of a Th1 to Th2 shift. Thus, these studies help to illustrate the complexity of GA's MoA, and provide validation of the experimental system.

Several of these pathways were similar to those observed in an earlier splenocyte study of similar design (Bakshi et al., 2013), despite that fact that these studies were conducted using different microarray platforms (Illumina versus Affymetrix), different pathway analysis platforms (Ingenuity Pathway Analysis versus DAVID enrichment), and different negative controls (medium versus mannitol). These include upregulated lymphocyte activation and downregulated hematopoietic cell lineage.

Downregulation of both *Il18* and *Il18r1* expression upon GA treatment has been previously reported (Bakshi et al., 2013). In the current study, in addition to these effects, a reciprocal effect was noted for probesets of the IL-18 inhibitor, *Il18bp*, upregulated by GA treatment. The fact that Polimunol consistently modulates *Il18* to a significantly different extent than GA, regardless of immunization agent, warrants further investigation as it has potential implications for both safety and efficacy. IL-18 is important for T helper cell differentiation, and IL-18 levels are higher in serum from multiple sclerosis patients versus controls, as well as acute versus stable multiple sclerosis (Nicoletti et al., 2001). IL-18 also induces IFNg expression and has been implicated in multiple sclerosis immunopathogenesis (Losy and Niezgoda, 2001). IL-18 genetic variants have further been shown to affect multiple sclerosis risk (Karakas Celik et al., 2014; Thompson and Humphries, 2007). Moreover, mice deficient for IL-18 are resistant to EAE (Shi et al., 2000).

A number of interferon-related genes are upregulated by Polimunol relative to GA in this system, in both the microarray data and qRT-PCR confirmation. Fig. 4c shows illustrated pathways of IFN Type I production (Trinchieri, 2010), with overlay indicating genes including *RIG-I*, *MDA5*, and *IRF7* upregulated by Polimunol versus GA. As indicated, upregulation of these genes would be consistent with increased type I interferon production.

At the pathway level, a variety of immune-related pathways are enriched among the differentially-expressed probesets, including "immune system process" and "response to virus," and differential expression is seen for multiple genes affecting interferon signaling (e.g., leading to significant enrichment of the RIG-I-like receptor signaling pathway), together raising serious concerns for safety and efficacy. Chronic long-term changes in expression of key genes, regardless of the magnitude of fold change, could impact pathways relevant to multiple sclerosis.

APCs play a central role in the MoA of GA, by presenting GA to T cells during priming at the periphery, by possibly also introducing GA-specific T cells to auto-antigens in the brain (though such cross-reactivity is not required for benefit), and by changing cytokine secretion profile in response to GA. GA shifts monocytes to a type II, anti-inflammatory state marked by increased production of anti-inflammatory cytokines (e.g., IL-10) and decreased production of pro-inflammatory cytokines (e.g., IL-12) (Weber et al., 2007).

Hundreds of genes are differentially expressed in human monocytes following activation with Polimunol compared with activation with GA. This stands in sharp contrast to the lack of differences between the three different lots of GA tested in parallel, under blinding, in the same experiment (Supplementary Table 16). The fact that the differentially-expressed genes are enriched in key biological pathways such as "immune response" supports the biological relevance of the observed differences. Many of the pathways enriched among the differentially-expressed genes are relevant to GA's MoA, such as cytokine-cytokine receptor interactions, and relevant to potential safety concerns, such as inflammatory response and response to lipopolysaccharide pathways.

The response to lipopolysaccharide pathway was also enriched among genes differentially expressed by another purported generic, Probioglat (Kolitz et al., 2015). Out of the 137 pathways enriched among probesets upregulated by Polimunol relative to GA, 37 of these pathways were also enriched in the prior comparison with Probioglat (out of 64 total pathways seen for Probioglat). Of note, the introduction of Probioglat in Mexico was associated with a 3-fold increase in adverse events and a 7-fold increase in relapses (Kolitz et al., 2015).

Protein-level measurements in THP-1 cells supported the gene-level differences. Secretion of inflammation-related proteins (including IL-1b, MCP-1, RANTES, Gro-a, and MMP-9) was higher with Polimunol versus GA treatment, consistent with the microarray data described above.

Synthon's Polimunol glatiramoid is marketed in Argentina (as of May 2014) as a purported clinical equivalent to GA. Polimunol is believed to be the same product used in the only clinical trial conducted to date with the objective of demonstrating equivalence in terms of MRI imaging measurements (Cohen et al., 2014). Yet this clinical study (GATE) despite its large sample size, raises important questions regarding the reported clinical measures. For instance, given the sample size of GATE (Copaxone = 357, Placebo = 84), the probability of showing any level of annualized relapse rate (ARR) reduction (ie, clinical efficacy) compared with placebo, of the reference, well-established drug Copaxone, was high (>90%) based on results from multiple prior randomized, placebo-controlled studies with Copaxone® conducted by various sponsors over the last 2 decades. However, no effect in reducing ARR was recorded for the Copaxone arm versus placebo in the GATE study (an unlikely result expected to occur in only 10% of cases), attesting to the lack of assay sensitivity (ie, ability to distinguish an effective drug from an ineffective one). Additionally, the rate of adverse events leading to discontinuation was higher in the Synthon product arm than in the Copaxone arm (12 vs 5), which may be underlied to a certain extent by upregulation of pro-inflammatory pathways, a pattern

Fig. 6. Polimunol differs from GA treatment at the gene expression level. (a) *CYP1B1* is significantly upregulated by Polimunol relative to GA. (b) Pathways significantly enriched among top probesets differentially expressed between Polimunol and GA. (c) Volcano plot showing probesets driving the enrichment of the response to lipopolysaccharide pathway among probesets upregulated by Polimunol relative to GA. The dashed line indicates significance level of adjusted p value < 0.05.

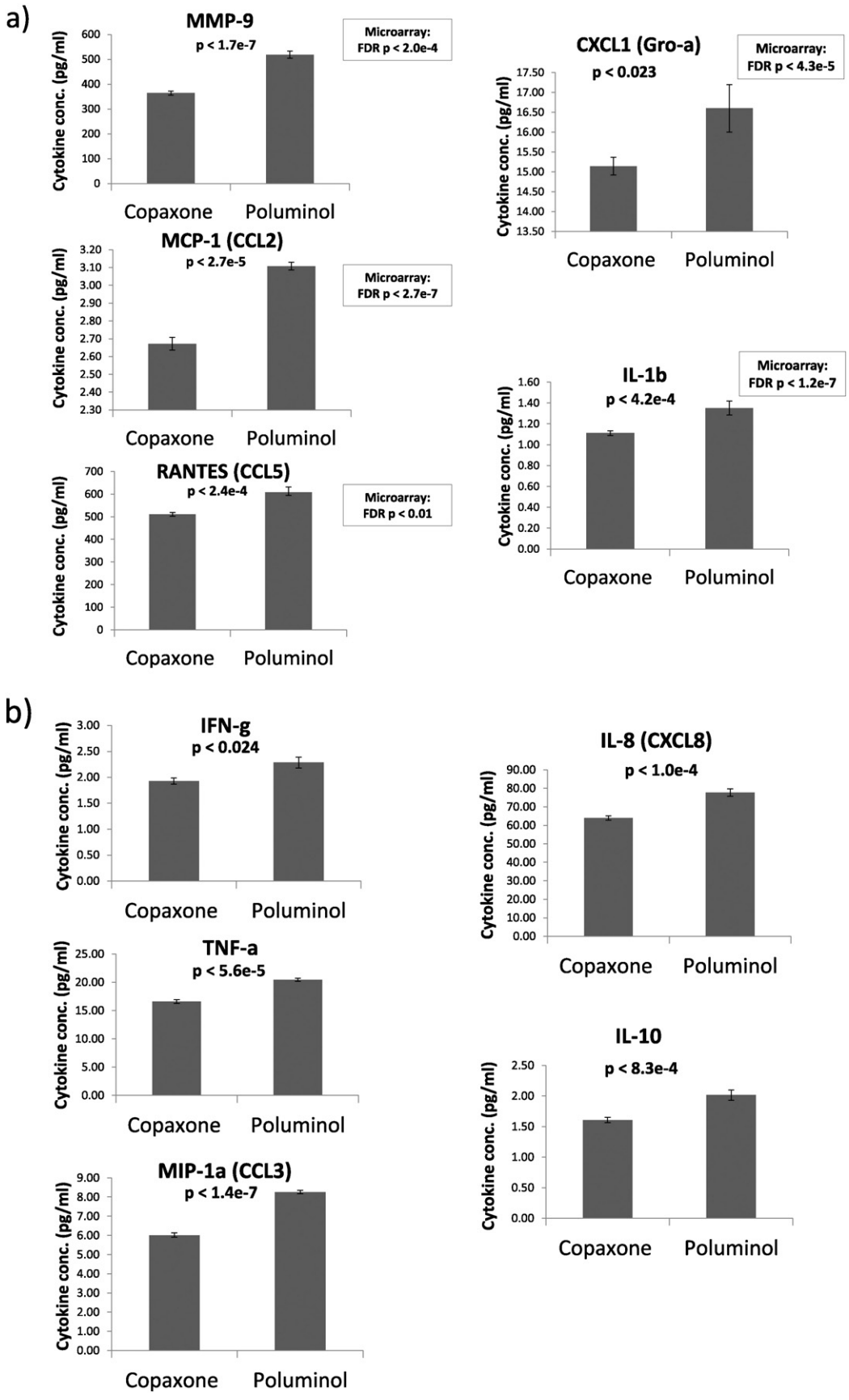


Fig. 7. Polimunol differs from GA treatment at the protein level. (a) Consistent with gene level data, levels of MMP-9, MCP-1 (CCL2), RANTES (CCL5), Gro-a (CXCL1), and IL-1b were higher with Polimunol versus GA treatment. (b) Levels of IFNg, TNFa, MIP1a (CCL3), IL-8 (CXCL8), and IL-10 were higher with Polimunol versus GA treatment.

similar to that reported for the Probioglat FOGA (Kolitz et al., 2015). Our findings demonstrate significant differences between GA and Polimunol at the mRNA, protein, and pathway levels.

4.1. Conclusions

Gene expression studies reported here add to the data around GA MoA, presenting comprehensive data from a human monocyte cell line in combination with a reciprocal-control mouse splenocyte model. Both of these studies employ a whole-genome based approach, looking across the entire expression array in an unbiased fashion, in model systems capturing differing, relevant aspects of GA's effects. The mouse splenocyte model used a novel reciprocal experimental design that enabled the modeling of patients switching to and from a differently manufactured glatiramoid, as well as comparing patients purely treated with GA with those purely treated with a differently manufactured glatiramoid. The resulting findings highlight the complexity of GA's MoA, modulating thousands of genes in dozens of different pathways. The biological impact is particularly sensitive to differences in composition and manufacturing (Weinstein et al., 2015). Significant differences were identified between the gene expression profiles modulated by Synthon's Polimunol and the profiles modulated by GA, in both human monocytes and mouse splenocytes. These data are in line with the significant differences between GA and Polimunol observed in multiple state-of-the-art physicochemical analyses (Weinstein et al., 2015). How best to determine whether a differently manufactured glatiramoid is as safe and effective as GA is the subject of active research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2015.11.020.

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